FIELD OF THE INVENTION

The subject of the present invention is a new genetic material encoding a new protein recognized by anti-Trypanosoma cruzi antisera, and it relates to the use of said gene and protein, especially for diagnostic, pharmaceutical and therapeutic purposes.

Trypanosoma cruzi is a flagellate protozoal parasite, a member of the order Kinetoplastida and of the family Trypanosomatidae, which is responsible for Chagas disease which affects naturally millions of persons, mainly in Latin America.

In vertebrate hosts, Trypanosoma cruzi is present in two forms: one which is mobile by means of its flagellum or trypomastigote and which does not divide; the other is aflagellate, or intracellular amastigote, which multiplies by binary division.

Transmission of the protozoan in man occurs through hematophagous insects of the family Reduviidae, during a blood meal followed by dejections at the site of the bite. The vector insect thus releases the infectious metacyclic trypomastigote forms which will colonize many cell types through the blood circulation. Trypanosoma cruzi infects cardiac and skeletal muscular cells, the glial cells and the cells of the mononuclear phagocytic system. After passive penetration into the host cell, the trypomastigote form of the parasite differentiates into the amastigote form, divides actively and then this is followed by a release of the trypomastigote forms, thereby causing a new cell invasion.

The insects will complete the parasitic cycle by ingesting, during a blood meal, the trypomastigote forms in the host. The latter differentiate into

epimastigote forms in the vector's middle intestine and finally into the infectious metacyclic trypomastigote forms in the posterior intestine.

Two phases can be distinguished in the Chagas disease: the acute phase and the chronic phase. The acute phase occurs after a transfusional, congenital or vectorial type contamination and lasts for a few weeks. It is characterized by a large number of parasites circulating in the blood and corresponds to an exponential division of the protozoan. The acute phase is most often asymptomatic. However, in infants contaminated by their mother, the acute phase, which is marked by an acute cardiopathy, may be critical. The chronic phase may extend over many years. In some individuals, this phase is asymptomatic. On the other hand, other patients have tissue lesions in the heart or digestive type manifestations. In any case, clinical diagnosis must always be confirmed by tests for the detection either of antibodies directed against the parasitic antigens, or of the parasite itself.

This disease is becoming a worldwide problem because of the contamination through blood transfusion. It was therefore becoming essential to have available diagnostic tests which make it possible to determine the presence of the parasite in individuals. Various serological tests include direct agglutination, indirect immunofluorescence (IIF), complement fixation tests (CFR), ELISA tests (Enzyme Linked Immunosorbent Assay). The Trypanosoma cruzi antigens used for the serological tests are obtained from a total lysate of the noninfectious stage of the parasite or from partially purified protein fractions. However, these

fractions do not allow antigens to be obtained in sufficient quantity and quality for the production of a reliable serological diagnostic test. Furthermore, the complexity of the parasite and the strain-to-strain antigenic polymorphism introduce an additional difficulty in the reproducibility of the different preparations. Finally, there are many risks of cross-reactivity with other protozoa, more particularly with Trypanosoma rangeli, a nonpathogenic parasite, and the family Leishmania. Another disadvantage of these techniques is the absence of determination of the disease phase which would allow a treatment from the onset of the acute phase.

In order to solve these various problems, it was envisaged to produce a serological diagnostic kit composed of recombinant proteins which would be specific for Trypanosoma cruzi.

Various research groups have screened libraries for expression of Trypanosoma cruzi genomic DNA or complementary DNA in the vector λgt11, using sera from patients suffering from Chagas disease. The λgt11 phage allows the insertion of foreign DNA of a maximum size of 7Kb into the EcoR1 site localized in the lacZ gene, under the control of the lac promoter. The product obtained is a recombinant protein used with beta-galactosidase, which is inducible by IPTG (isopropyl beta-D-thiogalactoside).

Various Trypanosoma cruzi genes, encoding proteins recognized by the Chagasic sera ~~where~~ were thus characterized. Among the recombinant antigens described, the H49 antigen may be mentioned (Paranhos et al., 1994 (1)). However, this antigen does not allow a

serological
~~sero-logical~~ detection sensitivity of 100% of the patients in the acute or chronic phase. It was therefore envisaged to combine the H49 antigen with the CRA ~~anti-gen~~ antigen (Cytoplasmic Repetitive Antigen) (Lafaille et al., (1989) (2)) but still without solving this problem.

SUMMARY OF THE INVENTION

The present inventors have identified and obtained for the first time a new genetic material encoding a new protein, recognized by anti-Trypanosoma cruzi antisera, which makes it possible to overcome the abovementioned disadvantages. The genetic material may be used to produce proteins or polypeptides for the production of diagnostic tests, or for the preparation of vaccinal or pharmaceutical compositions, or may itself either be used as a probe, or for the determination of specific probes which can be used in nucleic acid hybridization tests for the detection of Trypanosoma cruzi infections. Likewise, the protein or any corresponding polypeptide may be used for the production of antibodies specific for the parasite, for diagnostic or passive protection purposes.

DETAILED DESCRIPTION OF THE INVENTION

This gene was called Tc 100 by the applicant.

Consequently, the subject of the present invention is a DNA or RNA molecule consisting of at least one strand comprising a nucleotide sequence represented in the identifier SEQ ID ~~No.1~~ NO:1, or a sequence complementary or antisense or equivalent to said sequence identified in the identifier SEQ ID ~~No.1~~ NO:1, and especially a sequence having, for any succession of 100 contiguous monomers, at least 50%, preferably at least 60%, or better still at least 85% homology with said sequence.

Nucleotide sequence is understood to mean either a DNA strand or its complementary strand, or an RNA strand or its antisense strand or their corresponding complementary DNAs. The DNA sequence as represented in the identifier SEQ ID ~~No.1~~ NO:1 corresponds to the messenger RNA sequence, it being understood that the thymine (T) in the DNA is replaced by a uracil (U) in the RNA.

According to the invention, two nucleotide sequences are said to be equivalent in relation to each other, or in relation to a reference sequence if, functionally, the corresponding biopolymers can play essentially the same role, without being identical, with respect to the application or use considered, or in the technique in which they are involved; two sequences obtained because of the natural variability, especially spontaneous mutation, of the species from which they were identified, or because of induced variability, as well as homologous sequences, homology being defined below, are especially equivalent.

Variability is understood to mean any spontaneous or induced modification of a sequence, especially by substitution and/or insertion and/or deletion of nucleotides and/or of nucleotide fragments, and/or extension and/or shortening of the sequence at at least one of the ends; a nonnatural variability may result from the genetic engineering techniques used; this variability may result in modifications of any starting sequence, considered as reference, and capable of being expressed by a degree of homology relative to the said reference sequence.

Homology characterizes the degree of identity of two nucleotide (or peptide) fragments compared; it is measured by the percentage identity which is especially determined by direct comparison of nucleotide (or peptide) sequences, relative to reference nucleotide (or peptide) sequences.

Any nucleotide fragment is said to be equivalent to a reference fragment if it has a nucleotide sequence which is equivalent to the reference sequence; according to the preceding definition, the following are especially equivalent to a reference nucleotide fragment:

a) any fragment capable of at least partially hybridizing with the complementary strand of the reference fragment,

b) any fragment whose alignment with the reference fragment leads to the detection of identical contiguous bases, in greater number than with any other fragment obtained from another taxonomic group,

c) any fragment resulting or capable of resulting from the natural variability of the species, from which it is obtained,

d) any fragment capable of resulting from the genetic engineering techniques applied to the reference fragment,

e) any fragment, containing at least 30 contiguous nucleotides, encoding a peptide homologous or identical to the peptide encoded by the reference fragment,

f) any fragment different from the reference fragment by insertion, deletion, substitution of at least one monomer, extension or shortening at at least

one of its ends; for example any fragment corresponding to the reference fragment flanked at at least one of its ends by a nucleotide sequence not encoding a polypeptide.

The invention moreover relates to DNA or RNA fragments whose nucleotide sequence is identical, complementary, antisense or equivalent to any one of the following sequences:

- that starting at nucleotide 1232 and ending at nucleotide 2207 of SEQ ID ~~No.1~~ NO:1

- that starting at nucleotide 1232 and ending at nucleotide 1825 of SEQ ID ~~No.1~~ NO:1

- ~~and~~ that starting at nucleotide 1266 and ending at nucleotide 2207 OF SEQ ID NO:1

and especially the DNA or RNA fragments whose sequence has, for any succession of 30 contiguous monomers, at least 50%, preferably at least 60%, or better still at least 85% homology with any one of said sequences.

The subject of the invention is also a protein, called PTc100 by the applicant, having an apparent molecular mass of about 100 kDa, which is recognized by anti-Trypanosoma cruzi antisera, or an immunological equivalent of this protein, and fragments thereof. The amino acid sequence of this protein is represented in the identifier sequence SEQ ID ~~No.2~~ NO:2

Immunological equivalent is understood to mean any polypeptide or peptide capable of being immunologically recognized by the antibodies directed against said Ptc100 protein.

The invention also relates to any fragment of the Ptc100 protein. A particular protein fragment has a

sequence starting at amino acid 323 and ending at amino acid 520 of the sequence defined in the identifier SEQ ID ~~No 2~~ NO:2, said fragment being specifically recognized by anti-Trypanosoma cruzi antisera; the invention also relates to any immunological equivalent of said fragment.

The Ptc100 protein and said protein fragments may contain modifications, especially chemical modifications, which do not alter their immunogenicity.

Moreover, the subject of the present invention is also an expression cassette which is functional especially in a cell derived from a prokaryotic or eukaryotic organism, and which allows the expression of DNA encoding the entire Ptc100 protein or a fragment thereof, in particular of a DNA fragment as defined above, placed under the control of elements necessary for its expression; said protein and said protein fragments being recognized by anti-Trypanosoma cruzi antisera.

Generally, any cell derived from a prokaryotic or eukaryotic organism can be used within the framework of the present invention. Such cells are known to persons skilled in the art. By way of examples, there may be mentioned cells derived from a eukaryotic organism, such as the cells derived from a mammal, especially CHO (Chinese Hamster Ovarian) cells; insect cells; cells derived from a fungus, especially a unicellular fungus or from a yeast, especially of the strain Pichia, Saccharomyces, Schizosaccharomyces and most particularly selected from the group consisting of Saccharomyces cerevisiae, Schizosaccharomyces pombe, Schizosaccharomyces malidevorans, Schizosaccharomyces

sloofiae, Schizosaccharomyces octosporus. Likewise, among the cells derived from a prokaryotic organism, there may be used, without this constituting a limitation, the cells of a strain of Escherichia coli (E. coli) or enterobacterial cells. A large number of these cells are commercially available in collections, such as ATCC (Rockville, MA, USA) and AFRC (Agriculture & Food Research Council, Norfolk, UK). The cell may also be of the wild-type or mutant type. The mutations are described in the literature accessible to persons skilled in the art.

For the purposes of the present invention, an E. coli DH5a cell (marketed by the company CLONTECH under the reference: C2007-1) is used.

The expression cassette of the invention is intended for the production of the PTc100 protein or for fragments of said protein which are produced by the abovementioned E. coli cell, and which are recognized by human antisera. Such antisera are obtained from patients who have contracted a Trypanosoma cruzi infection recently or long ago, and contain immunoglobulins specifically recognizing PTc100. Of course, the PTc100 protein can also be recognized by other antibodies, such as for example monoclonal or polyclonal antibodies obtained by immunization of various species with the natural abovementioned protein, the recombinant protein or fragments or peptides thereof.

PTc100 protein is understood to mean the natural Trypanosoma cruzi cytoplasmic antigen, or the antigen produced especially by the genetic recombination techniques described in the present application, or any fragment or mutant of this antigen, provided

that it is immunologically reactive with antibodies directed against the PTc100 protein of this parasite.

Advantageously, such a protein has an amino acid sequence having a degree of homology of at least 70%, preferably of at least 85%, and most preferably of at least 95% relative to the sequence identified in the identifier SEQ ID ~~NO:2~~ NO:2. In practice, such an equivalent can be obtained by deletion, substitution and/or addition of one or more amino acids of the native or recombinant protein. It is within the capability of persons skilled in the art to perform, using known techniques, these modifications without affecting immunological recognition.

Within the framework of the present invention, the PTc100 protein can be modified in vitro, especially by deletion or addition of chemical groups, such as phosphates, sugars or myristic acids, so as to enhance its stability or the presentation of one or several epitopes.

The expression cassette according to the invention allows the production of a PTc100 protein (having an amino acid sequence as specified above) and fragments of said protein, fused with an exogenous element which can help its stability, its purification, its production or its recognition. The choice of such an exogenous element is within the capability of persons skilled in the art. It may be especially a hapten, an exogenous peptide or a protein.

The expression cassette according to the invention comprises the elements necessary for the expression of said DNA fragment in the cell considered. "Elements necessary for the expression" is understood

to mean the elements as a whole which allow the transcription of the DNA fragment into messenger RNA (mRNA) and the translation of the latter into protein.

The present invention also extends to a vector comprising an expression cassette according to the invention. This may be a viral vector and especially a vector derived from a baculovirus, more particularly intended for expression in insect cells, or an adeno-virus-derived vector for expression in mammalian cells.

It may also be an autonomously replicating plasmid vector and in particular a multiplicative vector.

The present invention also relates to a cell derived from a prokaryotic or eukaryotic organism, comprising an expression cassette, either in a form integrated in the cellular genome, or inserted in a vector. Such a cell was previously defined.

The subject of the present invention is also a process for preparing a PTc100 protein, or fragments of said protein, according to which:

(i) a cell derived from a prokaryotic or eukaryotic organism, comprising the expression cassette according to the invention, is cultured under appropriate conditions; and

(ii) the expressed protein derived from the abovementioned organism is recovered.

The present invention also relates to one or more peptides, whose amino acid sequence corresponds to a portion of the sequence of the PTc100 protein and exhibiting, alone or as a mixture, a reactivity with the entire sera from individuals or animals infected with Trypanosoma cruzi.

The peptides can be obtained by chemical synthesis, lysies of the PTc100 protein or by genetic recombination techniques.

The invention also relates to monoclonal or polyclonal antibodies obtained by immunological reaction of a human or animal organism to an immunogenic agent consisting of the natural or recombinant PTc100 protein and fragments thereof, or of a peptide, as defined above.

The present invention also relates to a reagent for the detection and/or monitoring of a Trypanosoma cruzi infection, which comprises, as reactive substance, a PTc100 protein as defined above, or fragments thereof, a peptide or a mixture of peptides as defined above, or at least one monoclonal or polyclonal antibody as described above.

The above reagent may be attached directly or indirectly to an appropriate solid support. The solid support may be especially in the form of a cone, a tube, a well, a bead and the like.

The term "solid support" as used here includes all materials on which a reagent can be immobilized for use in diagnostic tests. Natural or synthetic materials, chemically modified or otherwise, can be used as solid supports, especially polysaccharides such as cellulose-based materials, for example paper, cellulose derivatives such as cellulose acetate and nitrocellulose; polymers such as vinyl chloride, polyethylene, polystyrenes, polyacrylate or copolymers such as polymers of vinyl chloride and propylene, polymers of vinyl chloride and vinyl acetate; styrene-based copoly-

mers, natural fibers such as cotton and synthetic fibers such as nylon.

Preferably, the solid support is a polystyrene polymer or a butadiene/styrene copolymer. Advantageously, the support is a polystyrene or a styrene-based copolymer comprising between about 10 and 90% by weight of styrene units.

The binding of the reagent onto the solid support may be performed in a direct or indirect manner.

Using the direct manner, two approaches are possible: either by adsorption of the reagent onto the solid support, that is to say by noncovalent bonds (principally of the hydrogen, Van der Walls or ionic type), or by formation of covalent bonds between the reagent and the support. Using the indirect manner, an "anti-reagent" compound capable of interacting with the reagent so as to immobilize the whole onto the solid support can be attached beforehand (by adsorption or covalent bonding) onto the solid support. By way of example, there may be mentioned an anti-PTc100 antibody, on the condition that it is immunologically reactive with a portion of the protein different from that involved in the reaction for recognizing the antibodies in the sera; a ligand-receptor system, for example by grafting onto the PTc100 protein a molecule such as a vitamin, and by immobilizing onto the solid phase the corresponding receptor (for example the biotin-streptavidin system). Indirect manner is also understood ~~under-stood~~ to mean the preliminary grafting or fusion by genetic recombination of a protein, or a fragment of this protein, or of a polypeptide, to one end of the

PTc100 protein, and the immobilization of the latter onto the solid support by passive adsorption or covalent bonding of the protein or of the polypeptide grafted or fused.

The invention also relates to a process for the detection and/or monitoring of a Trypanosoma cruzi infection in a biological sample, such as a blood sample from an individual or an animal likely to have been infected with Trypanosoma cruzi, characterized in that said sample and a reagent as defined above are placed in contact, under conditions allowing a possible immunological reaction, and the presence of an immune complex with said reagent is then detected.

By way of non-limiting example, there may be mentioned the sandwich-type detection process in one or more stages, as especially described in patents FR 2,481,318 and FR 2,487,983, which consists in reacting a first monoclonal or polyclonal antibody specific for a desired antigen, attached onto a solid support, with the sample, and in revealing the possible presence of an immune complex thus formed using a second antibody labelled by any appropriate marker known to persons skilled in the art, especially a radioactive isotope, an enzyme, for example peroxidase or alkaline phosphatase and the like, using so-called competition techniques well known to persons skilled in the art.

The subject of the invention is also an active immunotherapeutic composition, especially a vaccinal preparation, which comprises as active ingredient, a natural or recombinant PTc100 protein or fragments thereof, or the peptides identified above, the active ingredient being optionally conjugated with a pharma-

ceutically acceptable carrier, and optionally an excipient and/or an appropriate adjuvant.

The present invention also covers a pharmaceutical composition intended for the treatment or for the prevention of a Trypanosoma cruzi infection in man or in an animal, comprising a therapeutically effective quantity of an expression cassette, a vector, a cell derived from a prokaryotic or eukaryotic organism as defined above, a PTc100 protein according to the invention, or fragments thereof, or an antibody of the invention.

The subject of the present invention is also probes and primers specific for T. cruzi, and their uses in diagnostic tests.

The term probe as used in the present invention refers to a DNA or RNA containing at least one strand having a nucleotide sequence which allows hybridization to nucleic acids having a nucleotide sequence as represented in the identifier SEQ ID ~~No.1~~ NO:1 or a complementary or antisense sequence, or a sequence equivalent to said sequence, and especially a sequence having, for any succession of 5 to 100 contiguous monomers, at least 50%, preferably at least 60%, or even better at least 85% homology with SEQ ID ~~No.1~~ NO:1 with fragments thereof, or with a synthetic oligonucleotide allowing such a hybridization, nonmodified or comprising one or more modified bases such as inosine, 5-methyldeoxycytidine, deoxyuridine, 5-dimethylaminodeoxyuridine, 2,6-diaminopurine, 5-bromodeoxyuridine or any other modified base. Likewise, these probes may be modified at the level of the sugar, namely the replacement of at least one deoxyribose with a polyamide (P. E. NIELSEN

et al. (1991) (13)), or at the level of the phosphate group, for example its replacement with esters, especially chosen from esters of diphosphate, of alkyl and arylphosphonate and of phosphorothioate.

The probes may be much shorter than the sequence identified in the identifier SEQ ID ~~No:1~~ NO:1. In practice, such probes comprise at least 5 monomers, advantageously from 8 to 50 monomers, having a hybridization specificity, under defined conditions, to form a hybridization complex with DNA or RNA having a nucleotide sequence as defined above.

A probe according to the invention can be used for diagnostic purposes as capture and/or detection probe, or for therapeutic purposes.

The capture probe can be immobilized on a solid support by any appropriate means, that is to say directly or indirectly, for example by covalent bonding or passive adsorption.

The detection probe is labelled by means of a marker chosen from radioactive isotopes, enzymes especially chosen from peroxidase and alkaline phosphatase, and those capable of hydrolyzing a chromogenic, fluorigenic or luminescent substrate, chromophoric chemical compounds, chromogenic, fluorigenic or luminescent compounds, nucleotide base analogs, and biotin.

The probes of the present invention which are used for diagnostic purposes can be used in any known hybridization ~~techniques~~ technique, and especially the so-called "Dot-Blot" technique (Maniatis et al. (1982) (14)), the Southern Blotting technique (Southern E. M. (1975) (15)), the Northern Blotting technique, which is a

technique identical to the Southern Blotting technique but which uses RNA as target, and the sandwich technique (Dunn A.R..et al. (1977) (16)). Advantageously, the sandwich ~~tech-nique~~ technique is used which comprises a specific capture probe and/or a specific detection probe, it being understood that the capture probe and the detection probe must have a nucleotide sequence which is at least partially different.

Another application of the invention is a therapeutic probe for treating infections due to Trypanosoma cruzi, said probe being capable of hybridizing in vivo with the DNA or RNA of the parasite to block the ~~trans-lation~~ translation and/or transcription and/or replication ~~pheno-mena~~ phenomena.

A primer is a probe comprising 5 to 30 monomers, having a hybridization specificity, under predefined conditions, for the initiation of an enzymatic polymerization, for example in an amplification technique such as PCR (Polymerase Chain Reaction), in an elongation process such as sequencing, in a reverse transcription method and the like.

A preferred probe or primer will contain a nucleotide sequence chosen from the sequences SEQ ID ~~No:7~~ NO:7, SEQ ID ~~No:8~~ NO:8, SEQ ID ~~No:9~~ NO:9, SEQ ID ~~No:10~~ NO:10, SEQ ID ~~No:12~~ NO:12.

The invention also relates to a reagent for detecting and/or identifying Trypanosoma cruzi in a biological sample, comprising at least one probe as defined above, and in particular a capture probe and a detection probe, either or both corresponding to the above definition.

The invention therefore provides a process for selectively detecting and/or for identifying Trypanosoma cruzi in a biological sample, according to which the RNA, extracted from the parasite and optionally denatured, or the DNA, denatured extract, or the DNA obtained from reverse transcription of the RNA, is exposed to at least one probe as defined above and the hybridization of said probe is detected.

The invention will be understood more clearly upon reading the detailed description below which is made with reference to the accompanying figures in which:

DESCRIPTION OF THE FIGURES

Figure 1 represents the restriction map of the Tc100 gene, which map is deduced by Southern blotting of different fragments obtained after digestion of Trypanosoma cruzi DNA with restriction endonucleases.

Figure 2 is a schematic representation of the three overlapping regions of the Tc100 cDNA. The numbered arrows represent the oligonucleotides used as primers for the PCR amplification.

Example 1: Isolation of the Tc50 clone

An expression library was constructed from Trypanosoma cruzi genomic DNA fragments. The T. cruzi, strain G (YOSHIDA. N, (1983) (17)), DNA isolated from the metacyclic trypomastigote stage was digested with the enzyme DNase I. After selection of the fragments according to their size, they were ligated to synthetic EcoRI adaptors and cloned into the EcoRI site of lambda gt11 vector DNA (Young and Davis, 1983 (3); Cotrim et al., 1990) (4).

The clone, called Tc50 by the applicant, was isolated from the library by immunological screening with the aid of a mixture of sera from patients suffering from the chronic phase of the Chagas disease.

The Tc50 phage clone was purified, amplified and the insert was detected by the PCR ("Polymerase Chain Reaction") technique with the aid of the primers:

SEQ ID ~~No.3~~ NO:3 5'(GGTGGCGACGACTCCTGGAGCCCG)3' ~~>24,~~ 24

and

SEQ ID ~~No.4~~ NO:4 5'(TTGACACCAGACCAACTGGTAATG)3' ~~24,~~ 24

corresponding respectively to the nucleotide sequence of the left and right arms of the lambda gt11 phage DNA.

The 594 base pairs (bp) Tc50 DNA fragment, after EcoRI digestion, was subcloned into the expression vector pGEX (Pharmacia) linearized with EcoRI. The sequencing of the Tc50 clone DNA was carried out in this same vector with the aid of specific primers situated in 3' and 5' of the cloning site of pGEX, according to the chain termination technique (Sanger et al., 1977 (5)) and according to the manufacturer's procedure (USB-Amersham).

The nucleotide sequence of the 594 bp Tc50 fragment as well as its deduced amino acid sequence (198 aa) are represented in the identifiers SEQ ID ~~No.1.~~ NO:1 and SEQ ID ~~No.2,~~ NO:2 respectively. The nucleotide sequence of the 594 bp Tc50 fragment starts at nucleotide (nt) 1232 and ends at nucleotide 1825 of SEQ ID NO:1. The corresponding amino acid sequence starts at amino acid 323 and ends at amino acid 520 of SEQ ID ~~No.2.~~ NO:2

Example 2: Expression of the Tc50 clone in Escherichia coli

The construct pGEX-Tc50 (198 aa) synthesizes, in the bacterium DH5alpha, a protein fused with GST ("Glutathione S Transferase"), with an apparent molecular mass of 50 kDa, which is detected by SDS-PAGE polyacrylamide gel electrophoresis (SDS: sodium dodecyl sulfate) (Laemmli, 1970 (6)). The reactivity of the protein towards chagasic human sera was confirmed by the Western blotting technique (Towbin et al., 1979 (7)) with the aid of the same mixture of chronic phase chagasic sera which is used for screening the lambda gt11 library.

The soluble fraction of the recombinant GST-Tc50 protein obtained after lysis of the bacterial extracts by ultrasound was purified by affinity chromatography on a glutathione agarose column (Sigma), according to the method of Smith and Johnson, (1988) (8).

The antigenic properties of the recombinant GST-Tc50 antigen were tested by ELISA (Voller et al., 1975 (9)). ~~For that, microtiter~~ Microtiter plates (Maxisorp (trade ~~name), nunc)~~ name)) were sensitized with 100 ng/ml of GST-Tc50 antigen in 100 mM NaHCO3 (pH 9.6). After incubation with the patients' sera, the immune complexes were detected with the aid of a peroxidase-coupled anti-human IgG goat serum.

The results are presented in the accompanying table and show that the ~~entire~~ chagasic human sera tested ~~react~~ reacts specifically with the recombinant protein. No cross-reactivity was observed on 7 sera from

patients suffering from cutaneous or visceral leishmaniosis.

Example 3: Identification of the native T. cruzi protein having the antigenic determinants of the Tc50 clone

The detection of the native T. cruzi protein was performed after immunopurification of a mixture of chagasic human sera on the corresponding recombinant protein called PTc50 by the applicant.

The eluate of monospecific polyclonal antibodies which is obtained was used as probe, in Western blotting, on total protein extracts of different stages of the parasite. The selected antibodies specifically reacted with a protein of apparent molecular mass 100 kDa, called PTc100 by the applicant, which is expressed in all the tested strains of the parasite.

Example 4: Molecular analysis of the Tc100 gene -Southern blots

In order to establish the restriction map of the Tc100 gene (Figure 1), the T. cruzi, strain G, nuclear DNA was digested with different restriction endonucleases (BamHI, EcoRI, HindIII, PstI, PvuII, SacI, BamHI/EcoRI, BamHI/PvuII, EcoRI/HindIII, EcoRI/PstI, EcoRI/PvuII, EcoRI/SacI, PstI/SacI, PstI/PvuII, PvuII/SacI, PvuII/HindIII), separated on agarose gel and then transferred onto a nylon filter according to the Southern technique. The Southern blot hybridization was performed with the 594 bp ~~Tc 50~~ Tc50 DNA, which is a fragment of the Tc100 DNA described above,

radiolabelled with ^{32}P by random incorporation (Amersham).

- Cloning of a 3500 bp Tc100 genomic fragment

According to the results obtained by Southern blotting, the T. cruzi, strain G, genomic DNA was digested with the enzyme EcoRI and then separated on agarose gel. The EcoRI restriction fragments of about 3500 bp (Figure 1) were cloned into the vector lambda gt10 (Huynh et al., 1984 (10)) linearized by EcoRI. The phage clone containing the 3500 bp Tc100 genomic insert was isolated with the aid of the 594 bp radiolabelled probe described above. A 1041 bp fragment situated in the 3' region of the 3500 bp Tc100 genomic insert was sequenced. This sequencing was carried out gradually with the aid of the following primers:

SEQ ID ~~No.5~~ NO:5 5' (TCGGGCACTGACGCGGCG) 3' 18

SEQ ID ~~No.6~~ NO:6 5' (CTTATGAGTATTTCTTCCAGGGTA) 3' 24

The primer SEQ ID ~~No.5~~ NO:5 is situated in the previously sequenced portion of the 594 bp Tc50 fragment. The primer SEQ ID ~~No.6~~ NO:6 corresponds to the lambda gt10 phage primer.

This 1041 bp fragment, which starts at nucleotide 1403 and ends at nucleotide 2443 of SEQ ID ~~No.1~~ NO:1, has an open reading frame in phase with the sequence of the 594 bp Tc50 fragment.

Example 5: Cloning of the Tc100 cDNA

The cDNA was synthesized from total RNA from T. cruzi, strain G, epimastigots. The Tc100 cDNA was amplified by the PCR technique in three different fragments: a fragment A corresponding to the 5' region of 1459 bp, a fragment B corresponding to the central region of 942 bp, a fragment C corresponding to the 3' region of 1406 bp of the Tc100 cDNA, as schematically represented in Figure 2.

- Cloning of fragment A of the Tc100 cDNA

The total cDNA synthesized by AMV ("avian myeloblastosis virus") reverse transcriptase, with the aid of random hexanucleotides (Boehringer Mannheim), was amplified, by PCR, using the following pair of primers:

SEQ ID ~~No.7~~ NO:7 5'(AACGCTATTATTAGAACAGTT)3' 21, and

SEQ ID ~~No.8~~ NO:8 5'(TGCAGCAGCGGCAGAAGT)3' 18

SEQ ID ~~No.7~~ NO:7 corresponds to a portion of the consensus sequence of 35 nucleotides present in 5' of the mRNAs in trypanosomatides and called "spliced leader" (Parsons et al. 1984 (11)).

SEQ ID ~~No.8~~ NO:8 corresponds to the sequence complementary to a portion of the predetermined sequence of the 594 bp fragment, ~~and~~ which starts at nucleotide 1442 and ends at nucleotide 1459 of SEQ ID ~~No.1~~ NO:1, according to the coding strand numbering.

After verification by Southern blotting with the aid of the radiolabelled 594 bp probe previously described, the 1459 bp cDNA fragment corresponding to

the 5' region of Tc100 was cloned into the plasmid called pCRII (trade name) (Invitrogen), and sequenced. The sequence represented in the identifier SEQ ID ~~No.1~~ NO:1 starts at nucleotide 1 and ends at nucleotide 1459.

- Cloning of fragment B of the Tc100 cDNA

The T. cruzi total cDNA was amplified by PCR with the aid of the primers:

SEQ ID ~~No.9~~ NO:9 : 5'(CAGCCGACGGTAGCTGCGTCCT)3' 22

and

SEQ ID ~~No.10~~ NO:10: 5'(ACATAATGGCCTCGTTCACAC)3' 21

The sequence ID ~~No.9~~ NO:9 which corresponds to a portion of the 594 bp predetermined sequence of the Tc100 gene starts at nucleotide 1266 and ends at nucleotide 1287 of SEQ ID ~~No.1~~ NO:1.

The sequence SEQ ID ~~No.10~~ NO:10 corresponds to the sequence complementary to a portion of the 1041 bp previously described sequence of the Tc100 gene. ~~This~~ The portion complementary to ~~sequence~~ SEQ ID ~~No.10~~ NO:10 starts at nucleotide 2187 and ends at nucleotide 2207 of SEQ ID No.1, according to the coding strand numbering.

The fragment obtained, 942 bp in length, was cloned into the plasmid pCRII and sequenced. The sequence represented in the identifier SEQ ID ~~No.1~~ NO:1 starts at nucleotide 1266 and ends at nucleotide 2207.

- Cloning of fragment C of the Tc100 cDNA

In order to isolate the 3' portion of the Tc100 cDNA, the T. cruzi total cDNA was synthesized with the aid of the adaptor oligo$(dT)_{16}$ hybrid primer.

SEQ ID NO:11 5'(GACTCGCTGCAGATCGATTTTTTTTTTTTTTTT)3' 34

according to the RACE ("Rapid Amplification of cDNA Ends") procedure (Frohman et., 1988 (12)).

The 3' region of the Tc100 cDNA was amplified using the adaptor primer and the following pair of primers:

SEQ ID NO:12: 5'(CGAAGAGACCATGAACAACTT)3' 21

and

SEQ ID NO:13: 5'(GACTCGCTGCAGATCGAT)3' 18

The sequence SEQ ID NO:12 corresponds to a portion of the previously described 1041 bp sequence of the Tc100 gene, starting at nucleotide 1997 and ending at nucleotide 2017.

The sequence SEQ ID NO:11 corresponds to the arbitrary sequence of the adaptor represented in SEQ ID NO:11.

After checking by Southern blotting using the 1041 bp radiolabelled fragment previously described, the 3' fragment of the Tc100 cDNA, 1423 bp long, was cloned into pCRII and sequenced. The sequence represented in the identifier SEQ ID NO:1 starts at nucleotide 1997 and ends at nucleotide 3402.

The Tc100 complete cDNA, 3402 bp in size, was completely sequenced. It has a 2745 bp open reading frame and the deduced amino acid sequence is 915. The methionine codon is in position 266 and the stop codon in position 3011.

The Trypanosoma cruzi Tc100 gene encodes the new PTc100 protein of theoretical molecular mass 100 kDa.

Of course, since the DNA sequence of the gene has been fully identified, it is possible to produce the corresponding DNA solely by chemical synthesis, and then to insert the DNA into commercially available DNA vectors, using known techniques from the technology relating to genetic recombination.

TABLE

Disease	Sera	OD (492nm) detection threshold = 0.320
CHAGAS DISEASE	1	1.358 (+)
	2	1.278 (+)
	3	0.328 (+)
	4	0.404 (+)
	5	1.378 (+)
	6	1.059 (+)
	7	0.895 (+)
	8	1.791 (+)
	9	1.635 (+)
	10	1.427 (+)
	11	1.009 (+)
	12	1.743 (+)
	13	0.530 (+)
	14	1.035 (+)
	15	0.461 (+)
CUTANEOUS LEISHMANIOSIS	16	0.291 (-)
VISCERAL LEISHMANIOSIS (Kala azar)	17	0.071 (-)
	18	0.081 (-)
	19	0.279 (-)
	20	0.098 (-)
	21	0.067 (-)
	22	0.125 (-)

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